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STRUCTURAL CHANGES AND THE DIFFERENTIAL EXPRESSION OF OSTEOPROTEGERIN (OPG) AND RECEPTOR ACTIVATOR OF NUCLEAR FACTOR kB LIGAND (RANKL) IN SUBCHONDRAL BONE DURING THE DEVELOPMENT OF OSTEOARTHRITIS

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ABSTRACT

Osteoarthritis is a degenerative joint disease that is characterized by subchondral bone changes. These changes may be associated with bone remodelling, which involve coordinative functions between bone formation and resorption markers such as osteoprotegerin (OPG) and receptor activator of nuclear factor kB ligand (RANKL), respectively. Previous studies have shown that the structural changes were different between the subchondral bone plate and trabecular bone. However, the expression of bone formation and bone resorption markers underlying the differences between these two regions has yet to be determined. Hence, the present study aims to determine the structural changes and expressions of OPG/RANKL in the subchondral bone plate and trabecular bone during the development of osteoarthritis in Dunkin Hartley guinea pigs. Eighteen tibias were scanned using a micro-computed tomography at three different time points; 10, 20 and 30 weeks of age to determine their subchondral bone plate and trabecular bone thickness. Immunohistochemistry and histopathology methods were conducted to determine the expression of OPG/RANKL and microscopic osteoarthritis score, respectively. The results showed that subchondral bone plate and trabecular bone thickness were greater in the medial than the lateral side of the tibial plateau, and increased significantly with ageing ($p \leq 0.01$). In addition, across the time points, the OPG/RANKL ratio of medial Sbp initially increased, before decreasing at the final time point. In contrast, the OPG/RANKL ratio of medial Tb was initially constant but then decreased at 30 weeks of age. Interestingly, a significant correlation was observed between the subchondral bone plate thickness and OPG/RANKL ratio with microscopic osteoarthritis scores ($r = 0.5$, $p \leq 0.05$), suggesting that the subchondral bone plate plays an important role in the pathogenesis of osteoarthritis.

Key words: Osteoarthritis, osteoprotegerin, receptor activator of nuclear factor kb ligand, subchondral bone plate, trabecular bone

INTRODUCTION

Osteoarthritis (OA) is the most common form of deteriorative joint disease that leads to pain and walking disability (Zhang & Jordan, 2010). It is characterized by the thinning of the articular cartilage (Zamli *et al.,* 2014), thickening of subchondral bone plate and structural changes of trabecular bone (Bobinac *et al.*, 2013). These changes may occur as a result of the joint tissue remodelling (Sharma *et al.*, 2013). The bone

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remodelling is a process of two main sequential events; bone resorption and followed by bone formation. Bone resorption begins when osteoclasts break down the old bone. Meanwhile, the bone formation is rebuilding a new bone tissue by the osteoblasts (Logar *et al.,* 2007). A continuous bone remodelling is necessary to maintain the structural, metabolic and biomechanics properties of the bone (Zamli *et al.,* 2014). However, abnormal bone remodelling may lead to diseases such as osteoporosis (Lawrence, 2005) and OA (Huebner & Kraus, 2006).

Osteoprotegerin (OPG) and receptor activator of nuclear factor kb ligand (RANKL) are examples of biomarkers that can be used to determine the state of bone formation and bone resorption, respectively (Boyce & Xing, 2008). Both OPG and RANKL are expressed by osteoblasts, osteocytes and stromal cells (Fujiwara *et al.,* 2016). RANKL is essential for starting bone resorption through the enhancement of osteoclast differentiation and proliferation after binding to RANK. On the other hand, OPG plays an important role to protect the bone from being excessively resorpted by binding to RANKL, and as a consequence, it prevents RANKL from binding to RANK (Logar *et al.,* 2007). OPG/RANKL ratio is commonly used as an indicator of bone remodelling activities, in which a high ratio level of OPG/RANKL is an indicative of promoting bone formation while a low level ratio of OPG/RANKL favours bone resorption (Kwan Tat & Pelletier, 2008).

Previous studies have shown that the structural changes such as subchondral bone thickness were different between subchondral bone plate and trabecular bone (Mastbergen & Lafeber, 2011; Burr & Gallant, 2012). However, the expression of OPG and RANKL markers that underlie the differences between these two regions has yet to be determined. Hence, the present study aims to determine the structural changes and expressions of OPG/RANKL in subchondral bone plate and trabecular bone during the development of spontaneous OA in knee joints of Dunkin Hartley guinea pigs. It is expected that the structural changes and biochemical markers of bone remodelling are higher in the medial side of subchondral bone plate than the other side/region during the early development of OA in the Dunkin Hartley guinea pigs. If this hypothesis is proven to be true, the search for potential OA treatment involving bones needs to be targeting the specific regions of subchondral bone rather than the current generalised approach.

MATERIALS AND METHODS

Sample collection

Eighteen male Dunkin Hartley guinea pigs were purchased from the Laboratory Animal Research Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia at seven weeks of age and around 250 g of average weight. They were kept at the Animal House, Kulliyyah of Pharmacy, International Islamic University Malaysia (IIUM) before being euthanised at ten, 20 and 30 weeks of age by an administration of 1 mL/kg of pentobarbitone sodium via intramuscular injection. The knee joint was dissected and the soft tissues were removed. Then, the tibia was fixed in 10% of neutral buffered formalin (Merck, USA) solution and kept at room temperature for further analysis. These protocols were approved by the Institutional Animal Care and Use Committee (IACUC), IIUM (IIUM/IACUC Approval/ (10) (62)).

Micro-computed Tomography scanning and analysis

The tibia was scanned with Micro-computed tomography scanner (Skyscan 1176) at 180° rotation with a resolution of 9 μm in anterior posterior position. The voltage of the X-ray tube was set to 65 kV and the current at 153 μA with a 1.0 mm aluminium filter. For the image reconstruction, a modified Fieldcamp algorithm was used and undertaken by using the NRecon software (Version 1.6.1.5, Skyscan, Kontich, Belgium). The reconstructed images were then analysed by using Bruker SkyScan CT-Analyser (CTAn) software (Version 1.9.2.5, SkyScan, Kontich, Belgium). GIMP 2.8.18 software was used to superimpose a standard grid on each constructed image. The subchondral bone plate thickness was then determined by measuring the distance between the osteochondral junction to intersection between the subchondral bone plate and trabecular bone.

Histological process

The fixed tibias were decalcified in Decalcifier II (Leica Biosystem, USA) for about six hours. The tibias were then processed in a tissue processor, embedded in paraffin (Leica Biosystem, USA) and cut at 2.5 μm and 4.0 μm by using a microtome for immunohistochemistry and histopathology analysis, respectively.

Immunohistochemistry staining and analysis

The tissue sectioned underwent a pre-treatment process of deparaffinisation and rehydration according to the protocol at Lab Histopathology, Kulliyyah of Medicine, IIUM. Next, antigen retrieval was conducted using PT Link system (DAKO, USA) at pH9, 97°C for 20 minutes. Then the slides were washed using neutral buffer solution and incubated for 2 hours with primary antibody of rabbit polyclonal antibody OPG (1:1000) and RANKL (1:200) (Abcam Hong Kong Ltd.), followed by the secondary antibody (Dako REAL™ EnVision™/HRP, Rabbit/Mouse) for 30 minutes. The DAB (DAKO, USA) was used to develop brown colour and counterstained with haematoxylin. All these staining procedures were done using universal auto-stain (DAKO, USA). The sample appearing brownish yellow was considered as positive staining. The captured images (Olympus BX51M) were analysed by counting the percentage of positive stained cells over the total cells presented in the section using Image J software.

Histopathological analysis of articular cartilage

The sectioned tissues were stained with 0.04% toluidine blue prepared in 0.1 M sodium acetate buffer (pH 4) for 5 minutes. The images of stained sections were captured by using Olympus BX51M camera and the blindly scored by using Osteo-arthritis Research Society International (OARSI) system (Kraus *et al.,* 2010). The medial and lateral side of each articular cartilage were assessed and scored in terms of its structure, proteoglycan content, cellularity and tidemark integrity.

Statistical analysis

The data were statistically analysed by using the Statistical Package for the Social Sciences (SPSS) 12.0.1 software. The data were analysed by comparing between the medial and lateral sides and time points at ten, 20 and 30 weeks of age by using Paired T-test / Wilcoxon Signed Rank Test, and Analysis of Variance (ANOVA)/ Kruskal Wallis Test, respectively. Partial correlation was used to explore the relationship between Sbp and Tb thickness, OPG/RANKL ratio with AC degeneration, while controlling for weight and age. The data were presented as mean ± standard error of mean (SEM). A significant difference of $p \leq 0.05$ and $p \leq 0.01$ was denoted as * and **, respectively.

RESULTS

Micro-Computed Tomography

Figure 1 shows the subchondral bone plate in the medial side was significantly thicker than the lateral side of the tibia at 10 [medial: 146.67 ± 5.578 ìm, lateral: 130.83 ± 4.729 µm; p = 0.003], 20 [medial: 396.67 ± 27.467 ìm, lateral: 355.83 ± 31.369 µm; p: 0.001] and 30 weeks of age [medial: 630.83 ± 12.276 µm, lateral: 581.67 ± 14.53 µm; p: 0.006]. The subchondral bone plate thickness increased across the time points in both sides of the tibia. These changes were statistically significant ($p \leq 0.01$).

Unlike the subchondral bone plate thickness, there was no significant difference of trabecular bone thickness between sides at 10 [medial: 71.21

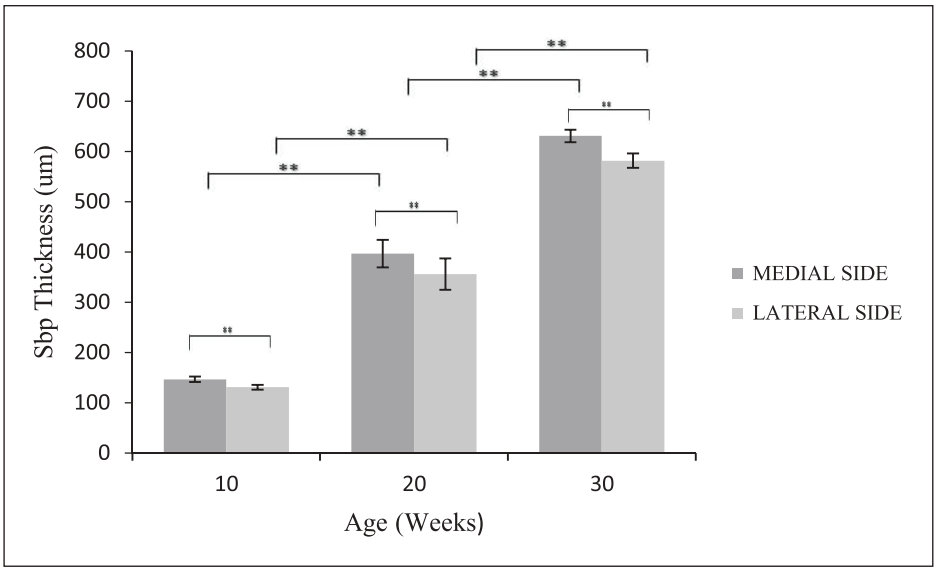

Fig. 1. Mean values of subchondral bone plate thickness in the medial and lateral side of Dunkin Hartley guinea pigs tibia at ten, 20 and 30 weeks of age. A significant difference of $p \leq 0.01$ was denoted as **. Error bars represent Standard Error Mean (SEM).

± 4.396 μm, lateral: 70.54 ± 3.529 μm], 20 [medial: 82.02 ± 1.796 μm, lateral: 81.41 ± 2.508 μm] and 30 weeks of age [medial: 118.16 ± 3.29 μm, lateral: 116.88 ± 2.269 μm] (Figure 2). However, these changes were statistically significant across the time points in both sides of the tibia ($p \leq 0.01$).

Immunohistochemistry

Figure 3 shows the representative micrographs of medial subchondral bone plate stained with OPG and RANKL at 10 (A and B), 20 (C and D) and 30 (E and F) weeks of age. Generally, the expression of OPG by the osteocytes in the medial subchondral bone plate was most pronounced at 20 weeks of age, while positively stained osteocytes by RANKL were predominantly observed at ten weeks of age and decreased with ageing.

Figure 4 shows the mean of OPG/RANKL ratios of positively-stained cells in the subchondral bone plate was initially higher in the medial than the lateral sides at 10 [medial: 0.92 ± 0.055, lateral: 0.88 ± 0.005] and 20 [medial: 1.27 ± 0.005, lateral: 1.02 ± 0.01] weeks of age. However, at 30 weeks of age it became lower in the medial than the lateral side [medial: 0.54 ± 0.01, lateral: 0.64 ± 0.01]. Across the time point, the mean of OPG/RANKL ratio in both sides of subchondral bone plate initially increased between ten to 20 weeks of age ($p > 0.05$). However, at the final time points, the OPG/RANKL ratio decreased, and even further, in the medial side of the subchondral bone plate ($p > 0.05$).

Unlike the subchondral bone plate, the expression of both OPG and RANKL decreased as age increased (Figure 5 A-F). The mean of OPG/RANKL ratios of positively-stained cells in the medial side of the trabecular bone was lower than the lateral side at 10 [medial: 0.605 ± 0.005, lateral: 1.00 ± 0.00] and 20 [medial: 0.59 ± 0.01, lateral: 0.795 ± 0.005] weeks of age. However, the trend changed at 30 weeks of age in which the mean of OPG/RANKL ratio was higher in the medial side than the lateral side [medial: 0.27 ± 0.00, lateral: 0.10 ± 0.005]. Across the time point, the mean of OPG/RANKL ratio in both sides of the trabecular bone slightly decreased between 10 to 20 weeks of age, but then drastically decreased at 30 weeks of age ($p > 0.05$).

Histopathology

Generally, the morphological changes of AC in the osteoarthritic knee joint was more prominent in the medial than the lateral side of the Dunkin Hartley guinea pigs tibia. The fibrillation of AC surface, loss of PG content and cellularity became worse as the age increased (Figure 7).

Figure 8 shows the total OARSI score was about the same for both medial and lateral side at ten weeks of age [medial: 5.33 ± 0.667; lateral: 5.00 ± 0.577]. However, as the age increased, the medial side shows higher score than the lateral side and reached statistical significance at 30 weeks of age [medial: 13.33 ± 1.53; lateral: 10.67 ± 1.53; p =

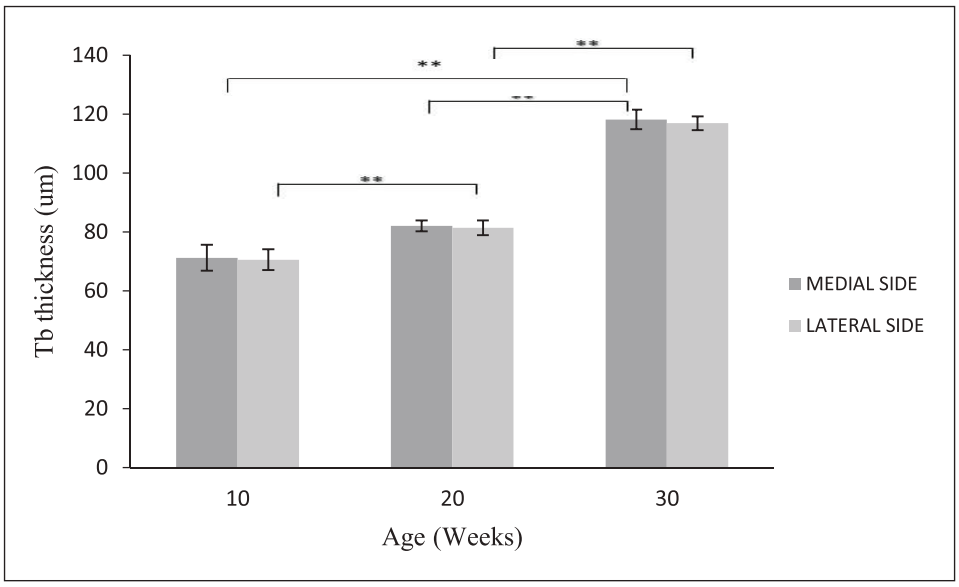

Fig. 2. Mean values of trabecular bone thickness in the medial and lateral side of Dunkin Hartley guinea pigs tibia at ten, 20 and 30 weeks of age. A significant difference of $p \leq 0.01$ was denoted as **. Error bars represent Standard Error Mean (SEM).

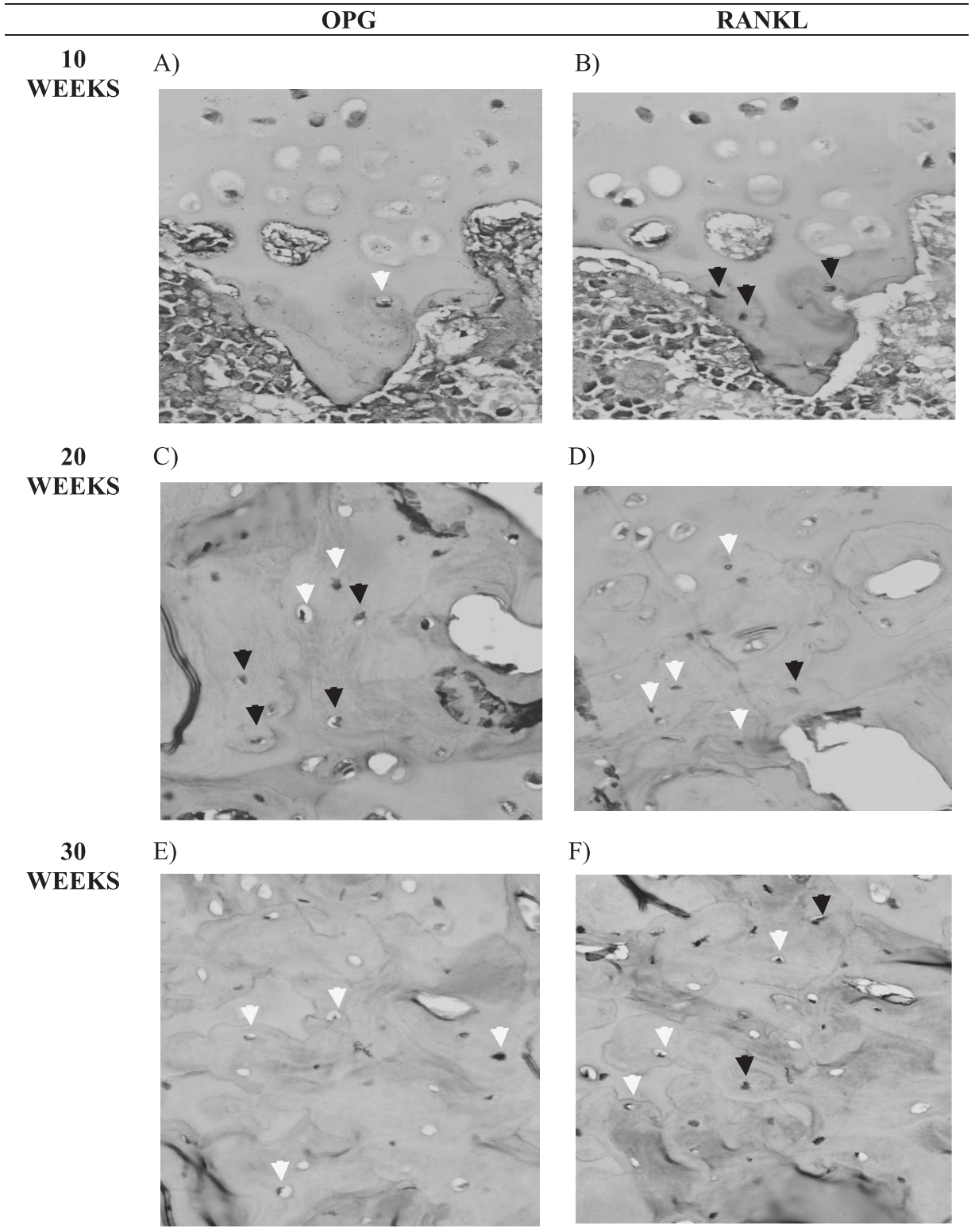

Fig. 3. Representative micrograph of subchondral bone plate stained with OPG and RANKL at ten (A and B), 20 (C and D) and 30 (E and F) weeks of age. The OPG/RANKL positive and negative osteocytes appear brownish (red arrow) and bluish (white arrow), respectively. Magnification: 200×.

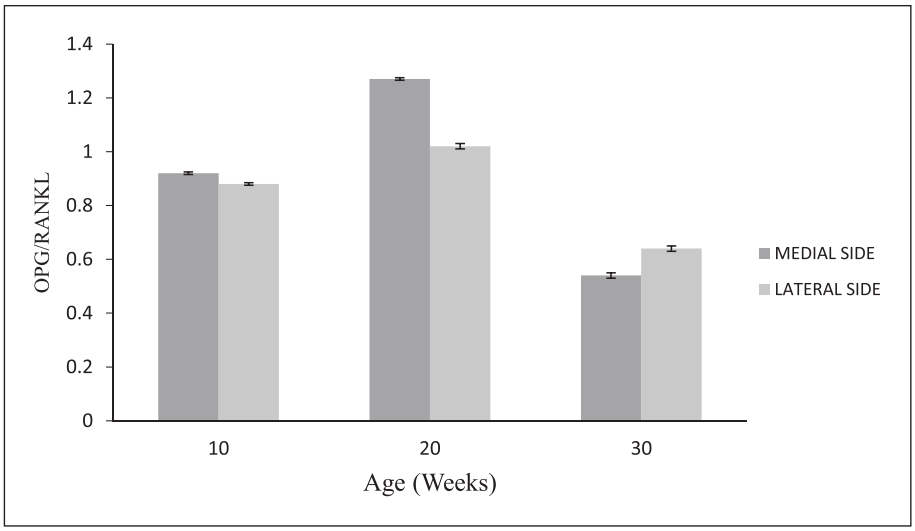

Fig. 4. Mean values of OPG/RANKL ratio of positively-stained osteocytes in the subchondral bone plate at the medial and lateral side of Dunkin Hartley guinea pigs tibia at ten, 20 and 30 weeks of age. Error bars represent Standard Error Mean (SEM).

0.015]. Across the time points, the OARSI scoring increased with ageing and was statistically significant between ten to 20 weeks of age (medial: $p = 0.008$; lateral: $p = 0.014$).

Correlation between the structural and molecular changes of subchondral bone and severity of OA

A significant correlation was observed between the total OARSI scores and the subchondral bone plate thickness ($r = 0.524$, $p = 0.037$) and OPG/RANKL ratio ($r = -0.669$, $p = 0.034$).

DISCUSSION

The present study highlights a number of important findings on the role of subchondral bone remodelling and its association with articular cartilage degeneration during the initiation and progression of spontaneous OA in Dunkin Hartley guinea pigs. The subchondral bone plate was significantly thicker in the medial than the lateral sides of the tibia and dramatically increased with ageing. Unlike the trabecular bone, the OPG/RANKL ratio of medial subchondral bone plate initially increased, before decreasing at 30 weeks of age. These changes progressed as a function of age and were significantly associated with the articular cartilage degeneration.

The data showed that the Sbp thickness was significantly greater in the medial than the lateral side of the tibia, which was consistent with previous studies in human and other animal models of OA (Goker *et al.,* 2000; Weng *et al.,* 2010; Zamli *et al*., 2014). Although not statistically significant, the medial side of the tibia also had more pronounced articular cartilage degeneration as compared to the opposite side of the tibia. As suggested by Intema *et al*. (2010), the medial side of the tibia has higher susceptibility to these structural changes due to the varus malalignment of the knee joint. This malalignment occurs as a result of unequal load distribution of the body weight between the two sides of the knee joint, resulting in higher susceptibility towards OA in the medial side of the tibia as it receives more stress than the opposite side (Patel *et al*., 2003).

Across the time points, the initial increase of OPG/RANKL ratio in the medial side of subchondral bone plate was attributed to the over expression of OPG, in respect to RANKL, that could tip the balance of subchondral bone remodelling toward bone formation. However, despite of continuous increase of subchondral bone plate thickness between 20 and 30 weeks of age, the OPG/RANKL ratio of medial subchondral bone plate decreased and became even lower than in the lateral side of the tibia. The present findings appear contradictory with those of Sniekers *et al*. (2012) and Bellido *et al*. (2010), who reported an early subchondral bone plate loss following a surgical induction of OA in dog and rabbit, respectively. The discrepancy in findings may be due to the different pathogenesis between spontaneous and induced animal models of OA.

In human, however, a significant lower OPG/RANKL ratio was observed in a patient with advanced knee OA as compared to healthy control subjects (Tat & Pelletier, 2008; Pilichou *et al.,* 2008), suggesting the subchondral bone resorption predominates over bone formation during the late stage of OA. Interestingly, the decrease of OPG/

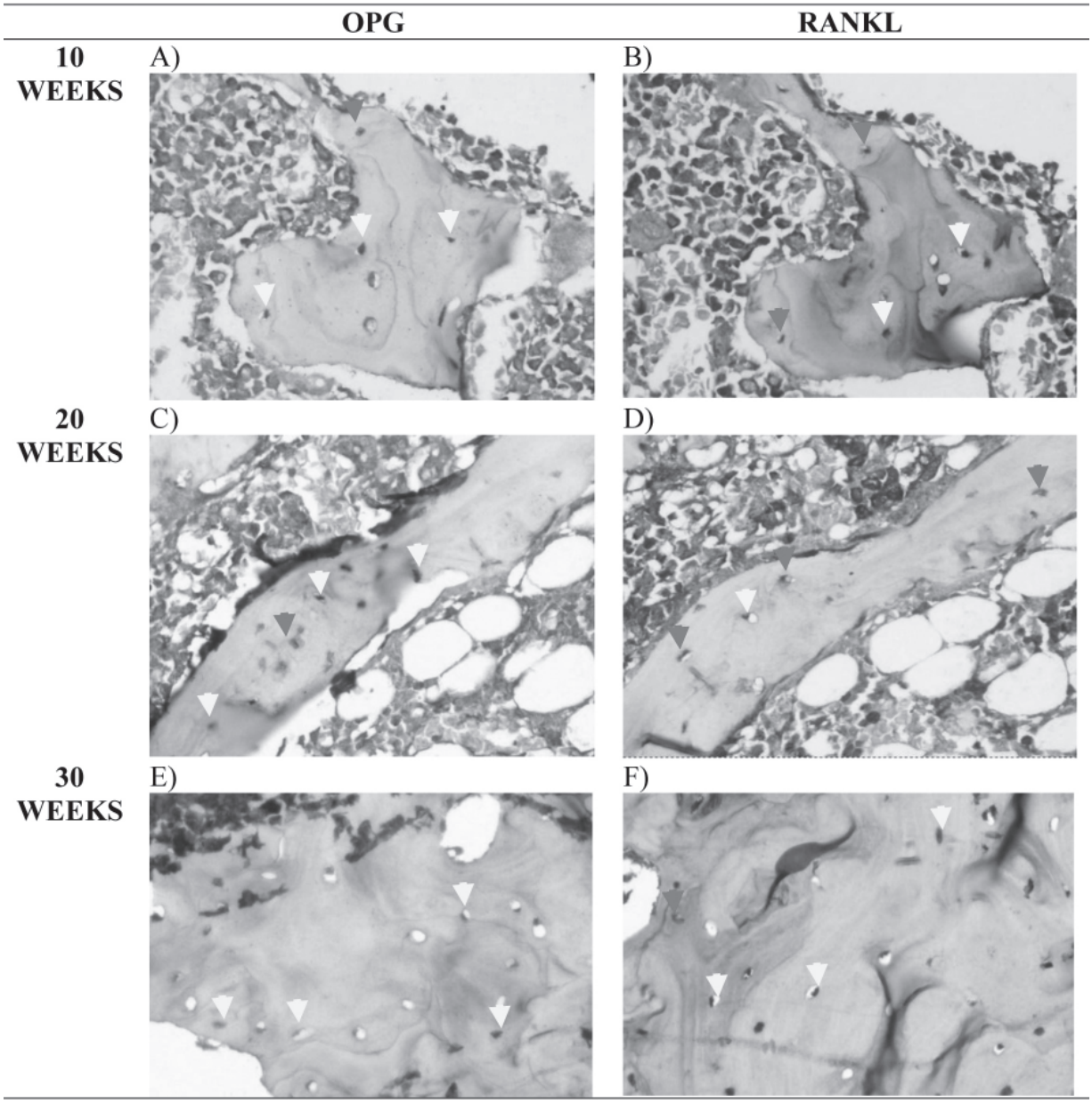

Fig. 5. Representative micrograph of trabecular bone stained with OPG and RANKL at ten (A and B), 20 (C and D) and 30 (E and F) weeks of age. The OPG/RANKL positive and negative osteocytes appear brownish (red arrow) and bluish (white arrow), respectively. Magnification: 200×.

RANKL ratio was not limited to osteoblasts in the osteoarthritic subchondral bone but also consistent with the expression of OPG/RANKL in OA chondrocytes (Kwan Tat *et al.,* 2009), the effect of which could be reversed by inhibiting the resorptive signals from the chondrocytes via celecoxib treatment (Moreno-Rubio *et al.,* 2010).

Intriguingly, although the present study indicates higher subchondral bone plate resorption between 20 and 30 weeks of age, the subchondral bone plate thickness continuously increased significantly until the final time point. To the knowledge of the researcher, this is the first study that demonstrates a significant inverse association between the subchondral bone plate thickening and OPG/RANKL ratio in spontaneous animal model of OA. It is hypothesised that, instead of thinning, the subchondral bone plate becomes porous at the late stage of OA, as a result of abnormal subchondral bone remodelling (Wen *et al.*, 2013; Hayami *et al.*, 2004; Bellido *et al.*, 2010). Further studies in human and animal models of OA are needed to confirm the above hypothesis.

This study has several limitations. First, lack of significant difference between side and time points in some analyses could be due to limited numbers of animal per time point. Second, the absence of growth-match disease free control for Dunkin Hartley

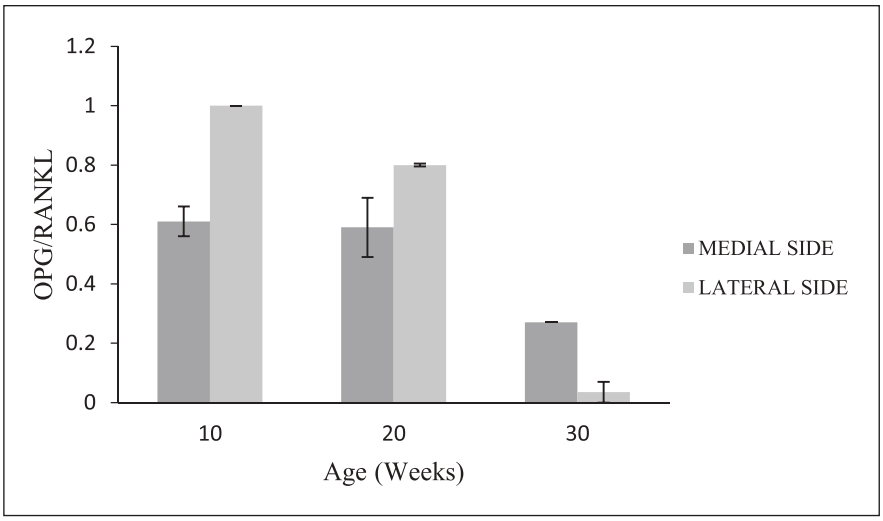

Fig. 6. Mean values of OPG/RANKL ratio of positively-stained osteocytes in the trabecular bone at the medial and lateral side of Dunkin Hartley guinea pigs tibia at ten, 20 and 30 weeks of age. Error bars represent Standard Error Mean (SEM).

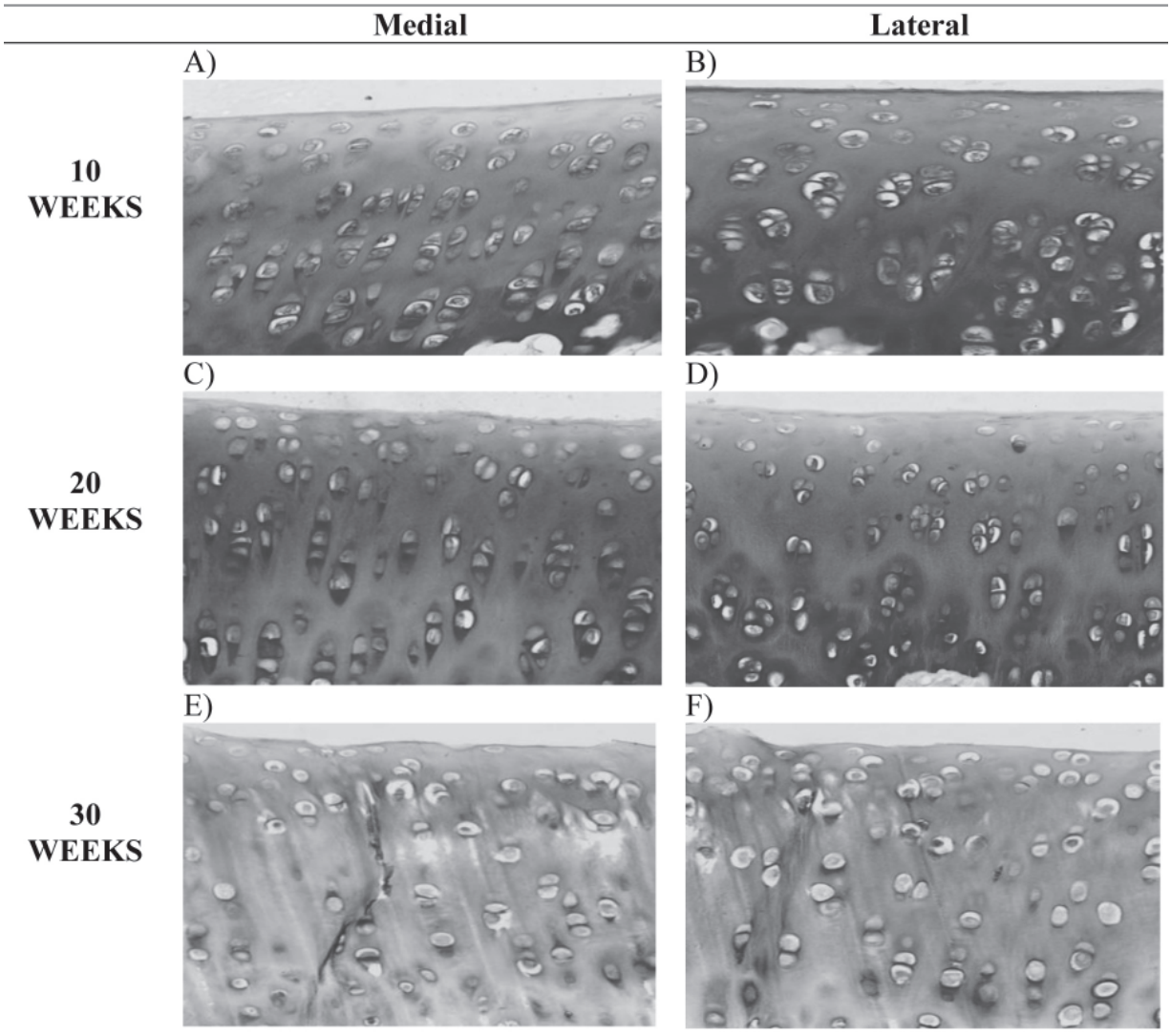

Fig. 7. Representative histological images of the medial and lateral articular cartilage of Dunkin Hartley tibia stained with toluidine blue at ten (A and B), 20 (C and D) and 30 (E and F) weeks of age. Magnification: 200×.

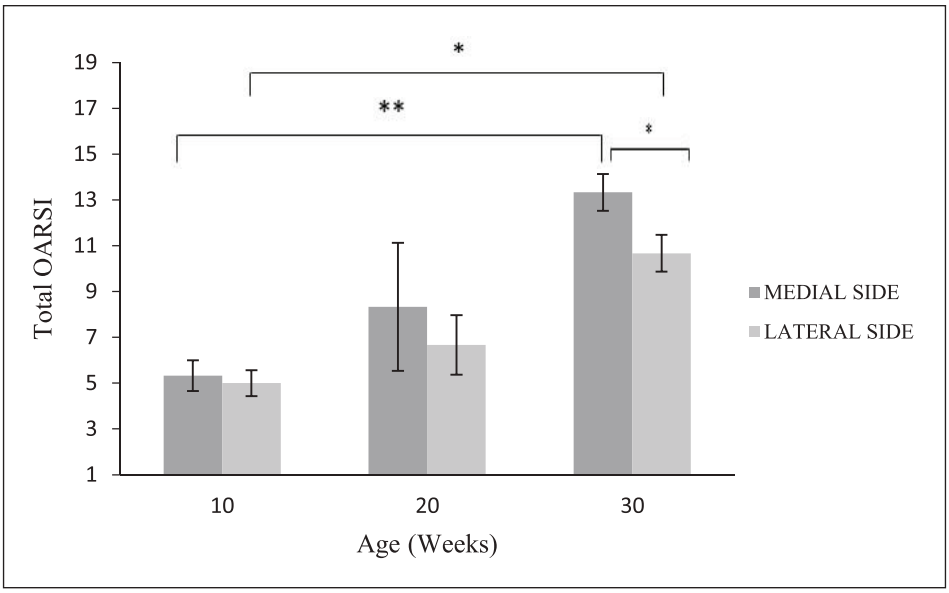

Fig. 8. Mean values of total OARSI score of articular cartilage in medial and lateral side of Dunkin Hartley guinea pigs at ten, 20 and 30 weeks of age. A significant difference of $p \leq 0.05$ and $p \leq 0.01$ was denoted as * and ** respectively. Error bars represent Standard Error Mean (SEM).

guinea pigs may lead to the wrong interpretation of subchondral bone changes that are due to growth or pathology. Nevertheless, this study used the lateral side of the tibia as the internal control because both sides of the tibia have similar growth rate.

CONCLUSION

Taken together, the present findings show that the structural and OPG/RANKL changes in the subchondral bone are more prominent in the medial than the lateral side of the Dunkin Hartley tibia. Unlike the trabecular bone, a significant correlation between the subchondral bone plate thickening and OPG/RANKL expression with articular cartilage degeneration suggests that the subchondral bone plate plays an important role in the pathogenesis of OA. Thus, the search for potential OA treatment involving bones needs to be targeting the specific regions of subchondral bone, and also consider the stage of disease.

ACKNOWLEDGEMENTS

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